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Forskolin Inducibility and Tissue-Specific Expression of the Fibronectin Promoter

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The mechanism of cyclic AMP (cAMP) induction of fibronectin (FN) in HT-1080 and JEG-3 cells differs (D. C. Dean, R. F. Newby, and S. Bourgeois, *J. Cell Biol.* 106:2159–2170, 1988). In the fibrosarcoma cell line HT-1080, induction requires both protein synthesis and a lag period of 12 to 24 h. In the choriocarcinoma cell line JEG-3, protein synthesis is not required and induction peaks before 24 h, declining thereafter. We show that the FN promoter is transcribed *in vitro* and that the transcripts initiate at the proper site. Based on transfection experiments with these cells and FN promoter constructions, a cAMP-responsive element (CRE) was identified between –157 and –188 base pairs upstream of the human FN gene. This sequence also conferred cAMP inducibility in both cell lines on the herpesvirus thymidine kinase promoter when it was placed upstream of a thymidine kinase-chloramphenicol acetyltransferase fusion gene. DNase I protection analysis and gel retardation experiments revealed that the CRE was bound by a protein(s) that was present in both HT-1080 and JEG-3 cells as well as in NIH 3T3 cells. Multiple protein-CRE complexes were resolved by gel retardation with extracts of both cell lines. Forskolin treatment of these cells did not alter qualitatively or quantitatively the pattern of CRE-binding proteins that was observed. The FN promoter was at least 10 times more active in HT-1080 than in JEG-3 cells, even though in JEG-3 cells both the rate of FN biosynthesis and the level of accumulated FN mRNA were greater than those in HT-1080 cells. The difference in promoter activity in HT-1080 and JEG-3 cells was mediated by sequences that were located between positions –510 and –56. Deletion of the FN promoter from positions –510 to –56 resulted in an ~30-fold decrease in promoter activity when this construction was transfected into HT-1080 cells, and similar results were observed in NIH 3T3 cells; however, less than a 2-fold effect was observed in JEG-3 cells. Results of these studies suggest that there is some degree of tissue specificity of FN gene expression and reveal that cAMP induction is mediated, in part, by the same element (CRE) in both HT-1080 and JEG-3 cells.

Fibronectin (FN) is a large extracellular matrix glycoprotein which serves as a substrate for cellular adhesion. As such, FN plays a role in cellular differentiation and migration, wound healing, hemostasis, and tumor metastasis (for reviews, see references 1, 14, and 31).

We have recently demonstrated that the rate of transcription of the FN gene is induced by cyclic AMP (cAMP) and agents which increase intracellular cAMP levels (8). The mechanism of this induction appears to be different in different cell lines. In normal fibroblasts and in the choriocarcinoma cell line JEG-3, cAMP induction of FN mRNA does not require protein synthesis and occurs rapidly, peaking before 24 h and decreasing thereafter. In the fibrosarcoma cell line HT-1080, cAMP induction of FN mRNA requires both protein synthesis as well as a lag period of 12 to 24 h; approximately 48 h is required for full induction. The amount of FN induction in HT-1080 (~27-fold) is also much larger than that in normal fibroblasts or JEG-3 cells (~5-fold). cAMP induction of FN expression is mediated by 5'-flanking sequences of the FN gene in HT-1080 and JEG-3 cells as well as in normal fibroblasts (8). It is of interest to determine whether similar promoter elements and nuclear proteins are required for this induction in cells in which there are apparent differences in the mechanism of induction.

The sequence TGACGTC, which is found at position

–170 of the human FN gene (7), has been identified in the cAMP-responsive element (CRE) of several genes (6, 9, 17, 24, 33). This sequence is necessary for cAMP induction but requires surrounding sequences for activity (9, 24). A 43-kilodalton nuclear protein binds to CRE, and its activity may be modulated by phosphorylation (23). The CRE is similar in sequence to the phorbol ester-inducible element (TRE) that is found in several genes (1 base pair [bp] difference), but the TRE is not responsive to cAMP (3, 16, 20, 21). The TRE is the binding site of transcription factor AP-1 (activator protein 1) or *c-jun* (3, 21) and a *c-fos* protein complex (29). There is evidence that AP-1 and *c-jun*, *c-fos*, the CRE-binding protein (CREB), and the product of a recently isolated gene *junB* (32) may belong to a family of related transcription factors (2, 4, 32).

In this study we identified a CRE in the 5'-flanking region of the human FN gene and demonstrated that this sequence mediates cAMP induction in both HT-1080 and JEG-3 cells.

MATERIALS AND METHODS

Cell culture and DNA transfections. Cells were grown in Dulbecco modified Eagle medium in the presence or absence of 10% fetal bovine serum at 37°C as described previously (8). The HT-1080 cell line (28) was subcloned, and a clone, HT-1080C, was used in this study (26). JEG-3 cells (19) were not subcloned. Cells were transfected by the calcium phosphate method described previously (8). As an internal con-

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trol, plasmid pRSV- β Gal (11) was cotransfected with the test plasmid DNA. The cells were incubated for 5 h in the presence of the precipitated DNA and then rinsed two times with phosphate-buffered saline to remove the calcium phosphate. Assays for β -galactosidase were done as described previously (11) and were used to normalize the amount of protein extract used for chloramphenicol acetyltransferase (CAT) assays (13). JEG-3 cells were treated with forskolin (1.8×10^{-5} M) for 24 h, and HT-1080 cells were treated for 40 h.

Plasmid constructions and DNA sequencing. The plasmid pFNCAT contains DNA sequences from +69 to approximately -1.6 kilobases (kb) of the human FN gene fused to the CAT gene (8). Deletions in the FN sequence were made by using the following restriction enzyme sites: *PvuII* at position -510, *NaeI* at position -122, and *SmaI* at position -56. The same sites and a *BstNI* site at position -222 were used to obtain the FN gene 5'-flanking fragments for placement upstream of the thymidine kinase (TK) promoter. The 3' end of the FN promoter fragments corresponded to either *PstI* (position +69) or *NaeI* (position +8) restriction sites. For cloning into the *HindIII* site of the TK-CAT plasmid (see Fig. 5), both the *HindIII* site in the vector and the *BstNI* site of the FN gene were blunt-ended with the Klenow fragment of DNA polymerase I (22).

DNA sequencing was carried out on fragments that were subcloned into the plasmid pGEM-4 (Promega Biotec). Dideoxy sequencing of supercoiled plasmids was done as described by the suppliers (Promega Biotec, Madison, Wis.).

In vitro transcription assays. Nuclear extracts from HeLa cells were prepared by the method of Dignam et al. (10). Transcriptions were carried out in a 25- μ l reaction volume containing 15 μ l of nuclear extract, 0.5 μ g of template DNA, and 100 μ M each of ATP, CTP, and GTP, with 30 μ M UTP, 5 μ Ci of [α - 32 P]UTP (600 Ci/mmol), and MgCl_2 added as indicated. Reactions were incubated for 30 min at 30°C in the presence or absence of α -amanitin, after which time they were terminated and processed as described previously (12). Radiolabeled RNA was then fractionated by electrophoresis in a 4% sequencing gel.

DNase I protection analysis. Nuclear extracts were prepared from HT-1080 and JEG-3 cells as described previously (25). Probes for DNase I protection were prepared from the vector pGEM-4 (Promega Biotec) containing the FN promoter sequence from positions +69 to -510 cloned between the *PstI* (+69 end) and the *SmaI* (-510 end) sites of the vector. The plasmid was digested with either *EcoRI*, which was adjacent to the *SmaI* site, or with *HindIII*, which was adjacent to the *PstI* site. The linearized plasmid was then labeled with 32 P by using polynucleotide kinase (22). Plasmid labeled at the *EcoRI* site (noncoding strand) was then cut with *HindIII*, and plasmid labeled at the *HindIII* site (coding strand) was digested with *EcoRI*. Labeled fragments were purified on polyacrylamide gels and used for DNase I protection assays as described previously (25).

Gel retardation assays. Gel retardation assays were done as described by Treisman (36), but with several modifications. Nuclear extracts were prepared as described above for the DNase I protection analysis. Extracts were incubated in 10% glycerol-25 mM Tris hydrochloride (pH 7.5)-100 mM KCl-5 mM spermidine-5 mM EDTA-1 mM dithiothreitol-1 μ g of herring sperm DNA in 15- μ l volumes with \sim 0.5 ng of 32 P-labeled oligonucleotide. Incubations were done for 30 min at room temperature, and the samples were loaded directly onto a 4% polyacrylamide gel run at \sim 4 V/cm. Gels were dried and autoradiographed.

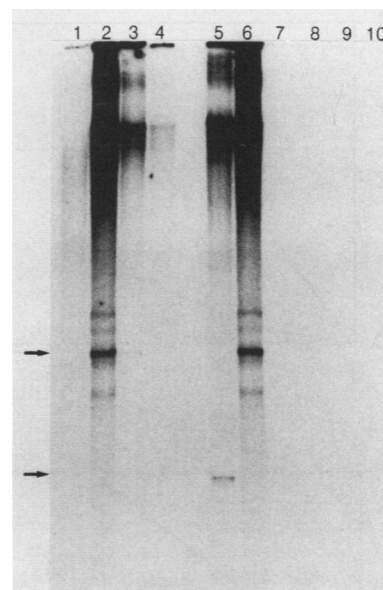


FIG. 1. In vitro transcription of the human FN promoter. Transcriptional assays were carried out with HeLa cell nuclear extracts as described in the text. The plasmid pFNCAT (-510) was used as the template DNA. The plasmid was digested with either *PvuII* (position +227) or *EcoRI* (position +329), and the site of transcriptional initiation of runoff transcripts was determined on a 4% sequencing gel by comparing the mobilities of the bands with that of a DNA sequence that was run in parallel. Lanes 1 to 4 (*EcoRI*), experiments done in the presence of various concentrations of MgCl_2 (lane 1, 1 mM; lane 2, 5 mM; lane 3, 7.5 mM; lane 4, 11 mM); lane 5 (*PvuII*), 5 mM MgCl_2 ; lane 6, same as lane 2; lanes 7 and 8 (*PvuII*), 5 mM MgCl_2 , 0.5 and 200 μ g of α -amanitin per ml, respectively; lanes 9 and 10 (*EcoRI*), 5 mM MgCl_2 , 0.5 and 200 μ g of α -amanitin per ml, respectively. The arrows indicate correctly initiated transcripts from *EcoRI*- and *PvuII*-cut plasmids.

RESULTS

In vitro transcription of the FN gene. The plasmid pFNCAT (-510) containing residues from positions -510 to +69 of the human FN gene fused to the CAT gene was digested with either *PvuII* or *EcoRI* and incubated with HeLa nuclear extracts for in vitro transcriptional runoff experiments (Fig. 1). An optimal MgCl_2 concentration of 5 mM was determined by titrating the amount of MgCl_2 in the reaction. Specific bands of 227 nucleotides (*PvuII*) and 329 nucleotides (*EcoRI*) were observed, indicating that in vitro transcription of the FN promoter initiates at the in vivo cap site. Each of these bands disappeared in the presence of low concentrations of α -amanitin, indicating that they were polymerase II transcripts.

Effect of the amount of FN promoter transfected on forskolin induction. The amount of the plasmid pFNCAT (-510) that was transfected into HT-1080 and JEG-3 cells was varied, and the effect on forskolin inducibility was examined. Basal expression increased in a linear fashion in both cell lines when the plasmid was increased; however, forskolin inducibility decreased with the amount of plasmid added in both cell lines (Fig. 2). In HT-1080 cells induction was 20-fold when 1 μ g of plasmid was transfected and declined to 2.8-fold when 15 μ g of plasmid was transfected. In JEG-3 cells induction was 12-fold with 3 μ g of plasmid; this declined to 2.6-fold with 20 μ g of plasmid. This suggests that at least one of the factors that is important for forskolin induction is present at a lower abundance or has a lower

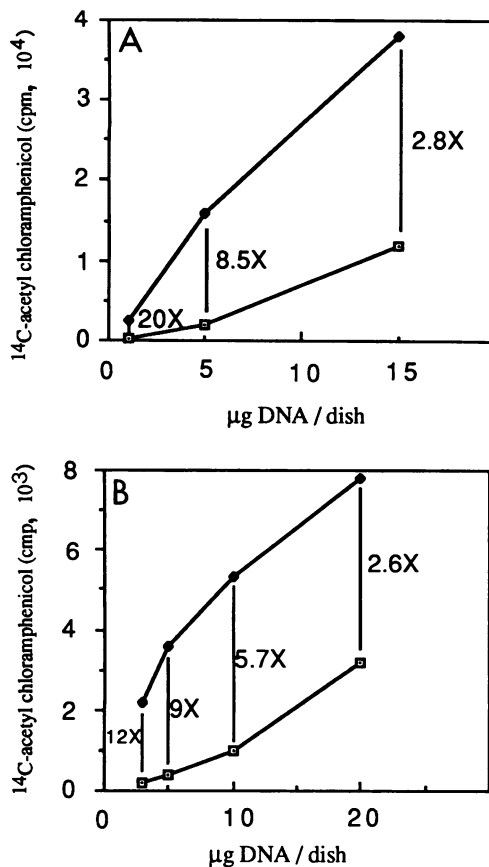


FIG. 2. Effect of FN-CAT copy number on forskolin induction. The amount of pFNCAT (–510) transfected into both HT-1080 and JEG-3 cells was varied. The total DNA was maintained at a constant level (15 µg for HT-1080 and 20 µg for JEG-3) by the addition of the plasmid vector pGEM-4. Forskolin was added at 1.8×10^{-5} M for 18 h in JEG-3 cells and 36 h in HT-1080 cells. Points were determined by cutting spots from the chromatograms and counting the radioactivity. The number of micrograms of DNA given corresponds to the amount of pFN-CAT (–510) added. Symbols: ■, Experiments done in the presence of forskolin; □, experiments done in the absence of forskolin. Numbers preceding the X represent the fold induction of CAT activity in each experiment. These experiments were repeated two times, with similar results obtained each time.

affinity than the factors that are necessary for basal expression of the FN gene.

The FN promoter is more active in HT-1080 than in JEG-3 cells. Cells were transfected with 5 µg of FN-CAT plasmid DNA. This amount of DNA allowed the detection of a measurable basal level of expression and forskolin inducibility (Fig. 2). The FN promoter appeared to be at least ~10 times more active in HT-1080 than in JEG-3 cells when FN promoter activity was compared with the activity of the simian virus 40 early promoter in each cell line (Fig. 3). Similar results were obtained if FN promoter activity was compared with the activity of either the Rous sarcoma virus (RSV) long terminal repeat (pRSV-βGal) or the herpesvirus TK promoter (TK-CAT) in both cell lines (data not shown). This was of interest since the amount of FN protein and mRNA was actually greater in JEG-3 cells. We have demonstrated previously that the half-life of FN mRNA in HT-1080 is shorter than that in normal fibroblasts (8). Therefore, the apparent discrepancy in promoter activity

and FN levels may be due to a longer mRNA half-life in JEG-3 cells compared with that in HT-1080 cells.

The difference in FN promoter activity in HT-1080 and JEG-3 cells was mediated by sequences between positions –56 and –510 (Fig. 3). Deletion of sequences between –1.6 kb and –510 bp resulted in an approximately threefold increase in expression in both cell lines, suggesting that a negative element may reside in this region. The FN promoter (position –510) was 12.5 times more active in HT-1080 cells than it was in JEG-3 cells. Deletion to –122 bp decreased this difference to only fivefold, while deletion to –56 bp resulted in equal activity in both cell lines. This indicates that in JEG-3 cells there is very little effect of sequences between –510 and –56 bp (<twofold) on transcription. However, in HT-1080 cells there was a strong dependence on this region. The region between positions –56 and –122 increased promoter activity 8.2-fold, while the whole region between positions –56 and –510 increased activity 30-fold. Similar effects were observed in NIH 3T3 cells (Fig. 4). Differences in expression of the FN promoter in HT-1080 and NIH 3T3 cells compared with that in JEG-3 cells reflected tissue specificity in the ability to respond to elements between positions –510 and –56.

Forskolin induction of the FN promoter. The FN promoter was inducible by forskolin in HT-1080 and JEG-3 cells (Fig. 3) and in NIH 3T3 cells (Fig. 4). It is also demonstrated in Fig. 4 that the FN promoter is induced by transforming growth factor β_1 and by treatment of serum-starved cells with serum in NIH 3T3 cells, suggesting that the regulation of FN expression in these cells was similar to that demonstrated previously for other cell lines (8). In HT-1080 cells forskolin inducibility was eliminated on deletion to –122 bp (Fig. 3). This eliminated the sequence TGACGTCA at position –170 which we identified previously (7) and which is similar to the core sequence of a CRE (6, 9, 17, 24, 33). In JEG-3 cells the deletion at –122 bp eliminated a portion of the forskolin induction; however, this construction was still induced threefold. Deletion to –56 bp eliminated forskolin induction in both cell lines. Examination of FN gene sequences between –122 and –56 bp revealed the sequence 5'-CCGCCCCGCC-3' at –119 bp on the coding strand which resembled the binding site for AP-2 of the metallothionein gene (5'-CCGCCCCGCG-3') that has been demonstrated to mediate forskolin inducibility (15). DNase I protection was observed in this region with extracts from JEG-3 cells but not from HT-1080 cells (see Fig. 6).

Effect of various segments of the FN gene 5'-flanking region on expression of the TK promoter. Various segments of the FN gene 5'-flanking region were placed upstream of the TK promoter (Fig. 5). The effect of these fragments on TK expression was analyzed in the presence or absence of forskolin (Fig. 5). A two- to fourfold increase in the basal level of TK expression was observed with the various fragments in each of the cell lines.

FN gene sequences between –122 and –510 bp were able to confer forskolin inducibility on the TK promoter. Most of this induction was eliminated when the region from –222 to –510 bp was used. However, the region between –122 and –222 bp was able to confer inducibility. A synthetic oligonucleotide containing the sequence from positions –157 to –188 was able to confer forskolin inducibility on the TK promoter, indicating that this sequence is a CRE. All of the FN segments shown in Fig. 5 were placed in the opposite orientation with respect to their natural orientations in the FN promoter. Similar results were obtained when the direct orientation of several of the constructions in HT-1080 cells

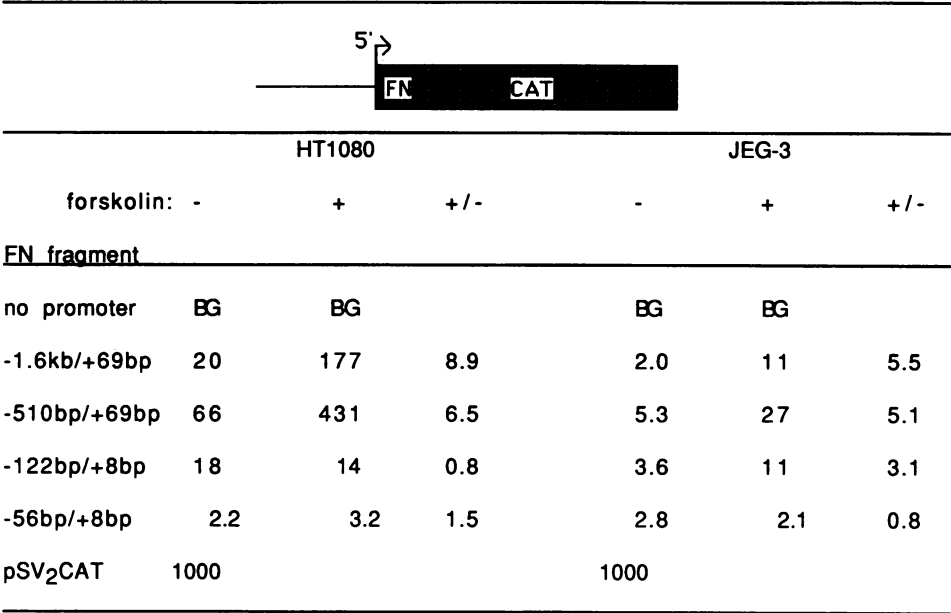


FIG. 3. Effect of successive 5' deletions on the activity and forskolin inducibility of the human FN promoter. A schematic diagram of the constructions is shown at the top. The numbers in the left column represent the amount of FN 5'-flanking sequence in the plasmid. The numbers representing CAT activity in the absence (-) or presence (+) of forskolin treatment were determined by counting spots from chromatography plates and normalizing the values to the activity of the simian virus 40 early promoter (which was set to be equal to 1,000). Each number is the average of three different experiments, and none of the values varied from the mean by more than 30%. The values presented under the +/- columns represent induction ratios calculated by dividing the values obtained in the presence of forskolin (+) by those obtained in the absence of the drug (-). BG, Not significantly above that of the background.

was used (positions -510 to -122 and positions -188 to -157) (data not shown).

Analysis of protein-binding sites on the FN promoter in vitro. Forskolin induction of the FN promoter was mediated through a CRE at -170 bp in each of the cell lines. Since

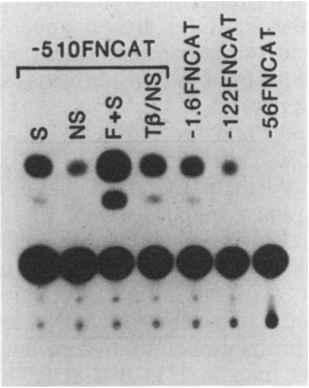


FIG. 4. Effect of forskolin (F), transforming growth factor β_1 (Tb), and serum treatment (S) and the effect of 5' deletions on expression of the FN promoter after transfection into NIH 3T3 cells. Serum-starved cells (no serum [NS]) were incubated for 36 h in the absence of fetal bovine serum, while serum-treated cells were incubated for 24 h in the absence of serum followed by treatment for 18 h with 10% fetal bovine serum. Treatment with forskolin was for 24 h, as described in the legend to Fig. 2, and T growth factor- β_1 (250 pM) treatment was for 18 h. Dishes (diameter, 10 cm) were transfected with 5 μ g of the indicated FN promoter plasmid. Numbers in front of FN-CAT indicate the length of the FN promoter fragments in each construct: -1.6, 1.6 kb; -510, 510 bp; -122, 122 bp; and -56, 56 bp. All experiments were done in the presence of 10% fetal bovine serum unless otherwise indicated.

protein synthesis is required for induction in HT-1080 cells but not in JEG-3 cells (8), it was of interest to determine whether the CREB was absent in untreated HT-1080 cells. DNase I protection was used to detect the binding of nuclear proteins to the FN promoter in vitro (Fig. 6). The region of the FN promoter around the CRE at -170 bp was protected with nuclear extracts from both HT-1080 and JEG-3 cells. Protection was observed in both strands, and hypersensitive sites were seen at the boundaries of the protected region. A similar amount of nuclear extract from each cell line was required for protection of the CRE, suggesting that CREB is present at similar concentrations in the two cell lines. In the case of HT-1080 nuclear extracts, DNase I protection in the region of the CRE covered two separate elements. Immediately 3' of the CRE was what appeared to be a nuclear factor 1-binding site (position -155). Protection of the CRE was eliminated on competition with an excess of an oligonucleotide containing the CRE, while protection of the putative nuclear factor 1 site was unaffected (data not shown). However, with nuclear extracts from JEG-3 cells, the CRE footprint did not extend 3' of position -160. This could have been caused by the absence of nuclear factor 1-binding activity in JEG-3 cells, but this possibility was not examined. One additional protected region was observed with extracts of both cell lines at about position -420. There was evidence for protected regions and hypersensitive sites in other regions as well, in particular, in the region from positions -53 to -115 (best visible on the coding strand in Fig. 6). This region was highly GC-rich and could correspond to several binding sites for transcription factor Sp1.

A gel retardation assay was done with an oligonucleotide containing the CRE (see Fig. 5 legend for a description of this oligonucleotide). Various amounts of extract from HT-1080, JEG-3, and NIH 3T3 cells were tested (Fig. 7). Each

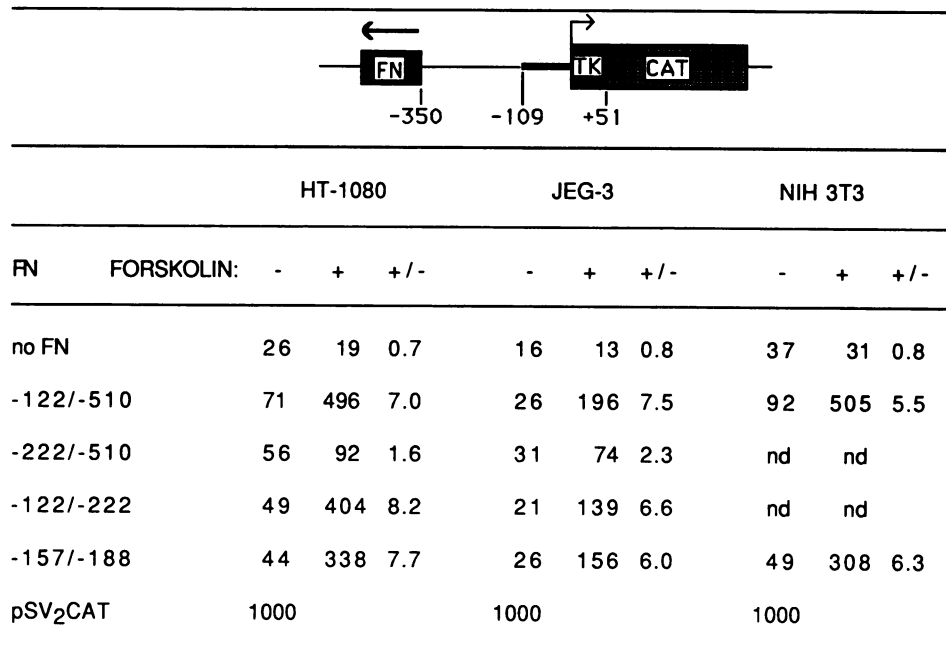


FIG. 5. Effect of segments of the FN gene 5'-flanking region on activity and forskolin inducibility of the TK promoter. A schematic diagram of the plasmids is shown at the top. FN DNA segments were cloned into the *Hind*III site of the TK-CAT plasmid at position -350 from the TK cap site (position +1). The numbers in the left column indicate the fragment of the FN gene 5'-flanking region present in each plasmid. The fragment from positions -157 to -188 was a synthetic oligonucleotide which corresponded to that region of the FN gene 5'-flanking sequences. CAT activity was normalized to that of the simian virus 40 early promoter (which was equal to 1,000), and the values obtained in the absence (-) or the presence (+) of forskolin and the induction ratios (+/-) were calculated as described in the legend to Fig. 3. This experiment was repeated at least five times. The values are averages of two experiments which were done at the same time and were representative of the other experiments. Each of the values was within 25% of the mean.

cell line contained a factor(s) which bound the CRE. Multiple complexes were seen with extracts from each cell line. Competition assays with an excess of cold oligonucleotide containing the CRE demonstrated that the binding was specific (Fig. 7A). In addition, when a 10-fold excess of competitor was used, two of the lower bands were still present, suggesting that these bands may represent the highest-affinity complexes; however, even these complexes were not able to bind in the presence of a 100-fold excess of competitor. Figure 7B shows a comparison of binding with extracts from HT-1080 and JEG-3 cells and the effect of forskolin treatment on binding. Multiple complexes were observed with extracts from both cell lines, and the migration of several of these complexes was similar in the two cell lines; however, there were at least two slowly migrating bands which were present with JEG-3 extracts that were not seen with HT-1080 extracts. There was also a rapidly migrating band which was present with HT-1080 extracts but not with JEG-3 extracts. There were no apparent differences in the abundance or mobility of the complexes in extracts from cells that were treated with forskolin. This was also true with extracts from NIH 3T3 cells (data not shown).

Sequence comparison of the 5' end and 5'-flanking region of the human and rat FN genes. The sequences of the 5' ends and the 5'-flanking regions of the rat and human FN genes are compared in Fig. 8. The two sequences were similar, and many of the differences which did occur resulted from the deletion or insertion of DNA segments. The CRE (position -170) and its adjacent sequences were identical in the rat and human FN genes. Immediately 3' of the CRE (centered at about -155 bp) there appeared to be a nuclear factor 1-binding site (30) which was similar in both sequences (CGGAN_{5,6}GCCAAAT). The other DNase I-protected re-

gions at -260 and -415 bp were also conserved in the two species.

DISCUSSION

Properly initiated transcripts were observed from the FN cap site when the FN-CAT fusion gene was used as a template for in vitro transcription. This suggests that CAT expression from the FN-CAT fusion gene is a reflection of FN promoter activity.

The FN promoter appeared to be at least 10 times more active in HT-1080 cells than in JEG-3 cells. This was surprising, initially, since JEG-3 cells have a greater rate of FN biosynthesis and a higher level of FN mRNA than do HT-1080 cells (8). We have demonstrated previously that the half-life of FN mRNA is shorter in HT-1080 cells than it is in normal fibroblasts (8). Therefore, this apparent discrepancy between promoter activity and the mRNA level in HT-1080 and JEG-3 cells probably results from a longer FN mRNA half-life in JEG-3 than in HT-1080 cells; however, direct measurements of the FN mRNA half-life have not been done in JEG-3 cells.

Deletion of FN gene sequences from positions -510 to -56 resulted in less than a 2-fold decrease of FN promoter activity in JEG-3 cells, while a similar deletion in HT-1080 or NIH 3T3 cells produced an ~30-fold decrease. The promoter activity in HT-1080 and JEG-3 cells was similar when the gene was deleted to position -56. The ability of the FN promoter to respond to sequences upstream of position -56 seemed to be tissue specific, since it was seen in both murine fibroblasts (NIH 3T3) and a human fibrosarcoma cell line (HT-1080) but not in a human choriocarcinoma cell line (JEG-3).

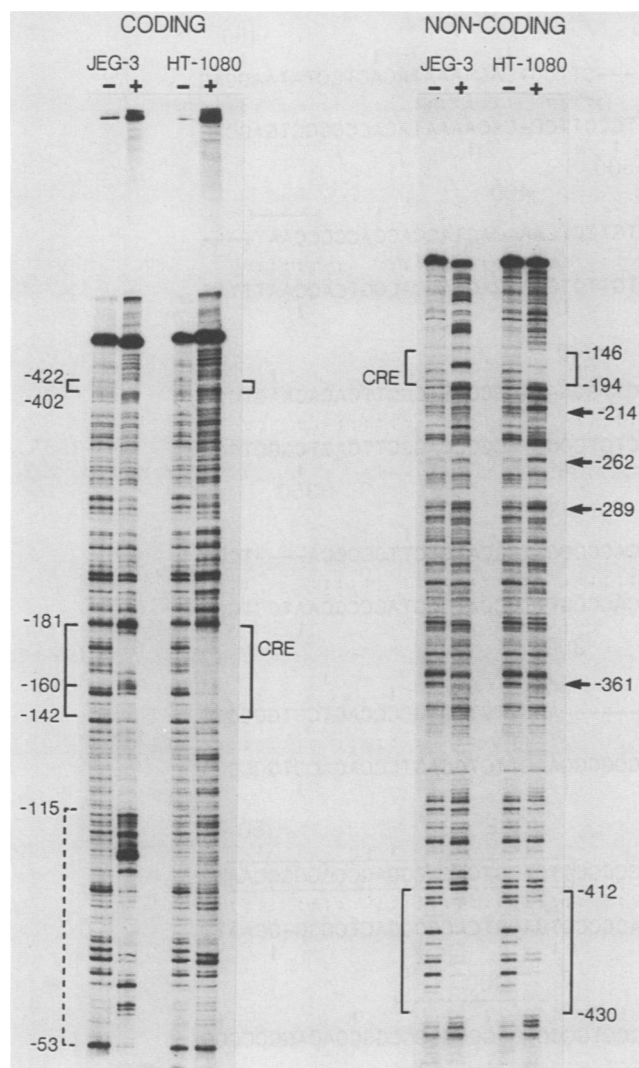


FIG. 6. DNase I protection assays of the human FN gene promoter. A DNA fragment of the 5'-flanking region of the FN gene from positions +69 to -510 was 32 P labeled on the 5' end of one strand 16 bp downstream of position +69 (*Hind*III site in vector) and was labeled on the other strand 18 bp upstream of position -510 (*Eco*RI site in vector). Labeled fragments were incubated in the absence (-) or presence (+) of nuclear extracts from JEG-3 (400 μ g) or HT-1080 (200 μ g) cells, treated with DNase I, and electrophoresed on 6% sequencing gels. Brackets indicate the boundaries of the protected regions (expressed in nucleotides from the site of initiation of transcription), which were calculated by using the fragments of *Msp*I-digested plasmid pBR322 as size markers (data not shown). The arrows and the dashed bracket indicate various regions with an altered DNase I sensitivity.

We have demonstrated previously that the mechanism of cAMP induction of FN is different in the fibrosarcoma cell line HT-1080 compared with that in the choriocarcinoma cell line JEG-3 (8). In most cell lines tested, the rate of FN biosynthesis is induced rapidly by cAMP or agents which increase intracellular levels of cAMP. A peak is reached before 24 h of treatment, and after this time expression decreases (to a point below the uninduced level in some cell lines). Inhibition of protein synthesis has no effect on the induction of FN mRNA in JEG-3 cells. However, in HT-1080 cells induction requires both a lag period of 12 to 24 h and protein synthesis (8). In this study we showed that a

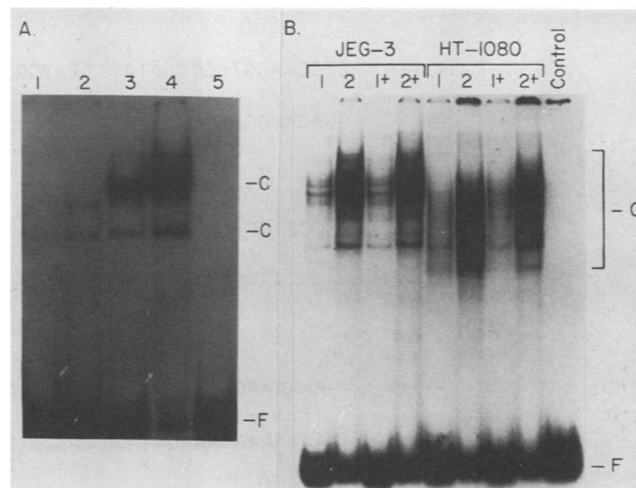


FIG. 7. Gel retardation assays of binding to the CRE in extracts from NIH 3T3, JEG-3, and HT-1080 cell lines; effect of forskolin treatment. (A) Extracts from NIH 3T3 cells were incubated in the presence of 32 P-labeled double-stranded oligonucleotide, which contained the CRE, and were subjected to electrophoresis on nondenaturing polyacrylamide gels as described in the text. Lane 1, 25 μ g of extract in the presence of a 100-fold excess of unlabeled CRE; lane 2, 25 μ g of extract from a 10-fold excess of unlabeled CRE; lane 3, 25 μ g of extract; lane 4, 51 μ g of extract; lane 5, no extract. (B) Effect of forskolin treatment on binding to the CRE in extracts from HT-1080 and JEG-3 cells. The reactions contained 5 (lane 1) and 25 (lane 2) μ g of protein extract. In lanes labeled with a plus sign, cells were treated with forskolin (as described in the legend to Fig. 2) before the extracts were made. Abbreviations: F, Uncomplexed oligonucleotide; C, oligonucleotide complexed with nuclear factors.

CRE is present at position -170 of the human FN gene and that this sequence can mediate cAMP induction in both HT-1080 and JEG-3 cells. Thus, it seems likely that there is a common pathway of cAMP induction in both cell types. In addition, a second sequence between positions -56 and -122 also contributes to cAMP induction in JEG-3 cells but not in HT-1080 cells. This site seems to function independently of the CRE. A sequence similar to the AP-2 site of the metallothionein gene, which has been demonstrated to mediate cAMP induction (15), is found in this region. It is not known, however, whether this is a functional AP-2-binding site; however, this region was protected in DNaseI protection assays with extracts of JEG-3 cells but not with extracts of HT-1080 cells. Since protein synthesis was required for induction in HT-1080 cells, one or more of the protein components of this pathway must be underrepresented in these cells. This factor does not appear to be CREB itself, since binding to the CRE was detected in both JEG-3 and HT-1080 cells. Multiple protein-CRE complexes (four to five bands) were observed with extracts from each of the cell lines. Several of the complexes had different electrophoretic mobilities when extracts from HT-1080 and JEG-3 cells were compared. The significance of these differences is unknown. Incubation of cells with forskolin before extracts were made did not alter the complexes that were formed from either cell. This indicates that the effect of forskolin is not to alter the affinity of CREB for the CRE, suggesting that the interaction of CREB with another transcription factor may be of importance and that forskolin may act to mediate such an interaction. However, we were not able to detect changes in the CRE-CREB complex, i.e., binding of an additional protein, when extracts from forskolin-treated cells were used.

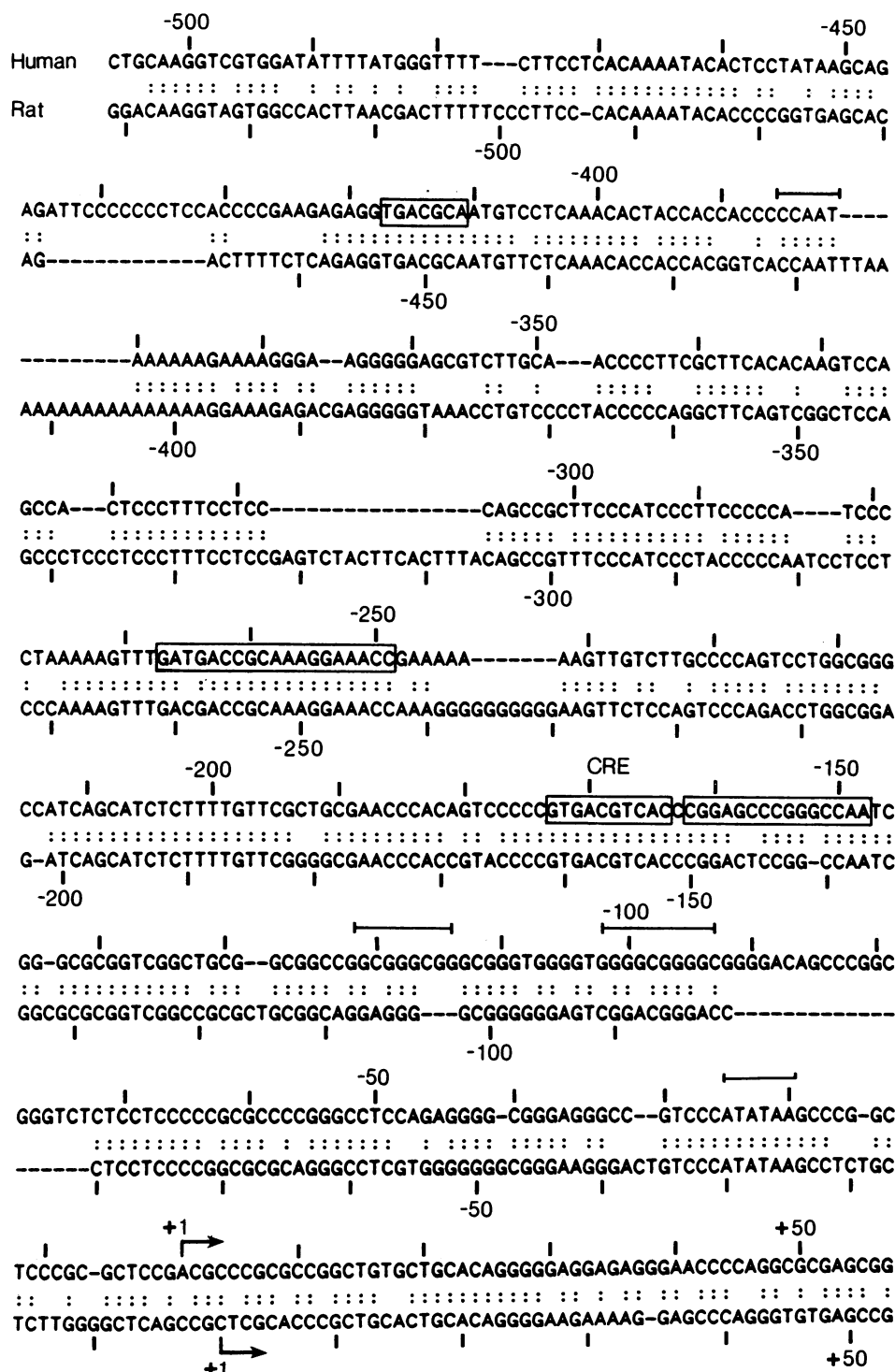


FIG. 8. Comparison of the 5' end and 5'-flanking regions of the human and rat FN genes. The human sequence to position -309 was from Dean et al. (7). The remainder of the sequence is reported here for the first time. The rat sequence is from Patel et al. (27). The alignment of the sequences was done with a software program (Intelligenetics IFIND) by using the align command. The parameters were as follows: Gap penalty, 4; DNA window, 20. Arrows indicate the start sites of transcription. Boxes are drawn around sequences that were protected in DNase I protection assays (Fig. 6). Bars are drawn over sequences which are similar to known regulatory elements.

It has been suggested that the activity of CREB is modulated by phosphorylation (23). Therefore, it is possible that either the level of the kinase responsible for this phosphorylation or a factor(s) that is involved in activation of this kinase may be suppressed in HT-1080 cells. Such a kinase or

factor would be, either directly or indirectly, inducible by cAMP. It has been demonstrated that the presence of an activated *N-ras* gene can inhibit the cAMP pathway (35). This suggests that in cells that are transformed by activated *ras* genes, a group of genes which is normally induced by

cAMP is not activated. Since HT-1080 cells contain an activated *N-ras* gene (5), it is possible that the differences observed in the mechanism of cAMP induction in HT-1080 and JEG-3 cells is due to the presence of this oncogene.

The effect of activated *ras* genes on cells seems to be similar to that of the phorbol ester 12-*O*-tetradecanoylphorbol-14-acetate (16). Transfection of cells with an activated *ras* gene (16) or treatment of cells with TPA (3, 21) results in induction of genes through the TRE or AP-1 or *c-jun* binding site. In transformed cells, two groups of transformation-sensitive genes were found: those that were activated and those that were suppressed. Genes such as those from simian virus 40, the polyomavirus enhancer, *fos*, collagenase, interleukin 2, and transin contain a TRE (3, 16, 18) and are constitutively activated on *ras* transformation. Genes which contain a CRE and depend on cAMP for activation, such as FN, may be suppressed or may remain at basal levels of activity. It has been demonstrated that transfection of activated *ras* genes into cells decreases the level of FN (34), and we have recently obtained indications that the rate of FN biosynthesis and the level of accumulated FN mRNA varies inversely with the number of activated *ras* genes in HT-1080 cells (L. Chandler and S. Bourgeois, manuscript in preparation).

Determination of the step in the cAMP induction pathway that is affected by activated members of the *ras* gene family may provide insight into one of the cellular functions of *ras* and may assist in defining the steps that are involved in cAMP induction.

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